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Na^+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437

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Bridges, Robert J., Ben B. Newton, Joseph M. Pilewski, Daniel C. Devor, Christopher T. Poll, and Rod L. Hall. Na^+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437. *Am J Physiol Lung Cell Mol Physiol* 281: L16–L23, 2001.—To test the hypothesis that Na^+ transport in human bronchial epithelial (HBE) cells is regulated by a protease-mediated mechanism, we investigated the effects of BAY 39-9437, a recombinant Kunitz-type serine protease inhibitor, on amiloride-sensitive short-circuit current of normal [non-cystic fibrosis (CF) cells] and CF HBE cells. Mucosal treatment of non-CF and CF HBE cells with BAY 39-9437 decreased the short-circuit current, with a half-life of ~45 min. At 90 min, BAY 39-9437 (470 nM) reduced Na^+ transport by ~70%. The inhibitory effect of BAY 39-9437 was concentration dependent, with a half-maximal inhibitory concentration of ~25 nM. Na^+ transport was restored to control levels, with a half-life of ~15 min, on washout of BAY 39-9437. In addition, trypsin (1 μM) rapidly reversed the inhibitory effect of BAY 39-9437. These data indicate that Na^+ transport in HBE cells is activated by a BAY 39-9437-inhibitable, endogenously expressed serine protease. BAY 39-9437 inhibition of this serine protease maybe of therapeutic potential for the treatment of Na^+ hyperabsorption in CF.

cystic fibrosis; Kunitz-type serine protease inhibitor; channel-activating protease; short-circuit current; primary cultures; epithelial sodium channel

CYSTIC FIBROSIS (CF) is characterized by abnormalities in anion secretion and Na^+ absorption in the airways (2, 3, 15, 22). Although a great deal is known about the regulation of anion secretion, the underlying mechanisms regulating airway Na^+ absorption are poorly understood. Recent studies (6, 27, 28) have demonstrated a novel extracellular serine protease-mediated signaling pathway for the modulation of amiloride-sensitive epithelial Na^+ channels (ENaCs). Amphibian and murine homologs of a cation channel-activating protease (CAP1) have been identified and are expressed in several epithelial tissues including kidney, prostate, salivary glands, colon and lung. *Xenopus* CAP1 (xCAP1) and murine CAP1 (mCAP1) share a 50% homology, whereas mCAP1 is 80% homologous with human prostasin (28). Prostasin is also expressed in epithelial tissues, including the lung (30, 31). Coexpression of xCAP1 (6, 27) or mCAP1 (28) with *Xenopus*, rat, or human ENaCs in *Xenopus* oocytes caused a severalfold increase in the amiloride-sensitive Na^+ current. Aprotinin, a bovine-derived serine protease inhibitor, blocked the activation of ENaCs by CAP1. Aprotinin also inhibited the baseline Na^+ transport in cultures of A6 cells, an amphibian renal cell line (27) and mpkCCD$_{C14}$ cells, a murine renal cell line (28).

The studies reported here were undertaken to test the hypothesis that Na^+ transport in human bronchial epithelial (HBE) cells is regulated by a protease-mediated mechanism. To test this hypothesis, we first investigated the effects of several protease inhibitors for their potential effects on the amiloride-sensitive short-circuit current (I_{sc}) in HBE cells. The results from these initial studies demonstrated that aprotinin inhibited HBE cell Na^+ transport. In contrast, two other serine protease inhibitors, soybean trypsin inhibitor (SBTI) and α_1-antitrypsin (α_1-AT), did not inhibit HBE cell Na^+ transport. The unique feature of aprotinin that defines the protease-inhibitory site of this bovine protein is the Kunitz domain (11, 12). A search for a human Kunitz-type serine protease inhibitor was made and led to the development of the recombinant protein BAY 39-9437. The studies reported here demonstrate the inhibitory effects of BAY 39–9347 on non-CF and CF HBE cell Na^+ absorption.

METHODS

Primary cultures of HBE cells. HBE cells were obtained from excess pathological tissue remaining after lung transplantation under a protocol approved by the University of Pittsburgh (Pittsburgh, PA) Investigational Review Board. Tissue expressing wild-type CF transmembrane conductance regulator (CFTR) was obtained after lung transplantation for a variety of pathological conditions including bronchiectasis,

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Table 1. *CFTR genotype of patients used to investigate the effects of BAY 39-9437*

Patient No.	Genotype
1	ΔF508/R560T
2	ΔF508/621 + 1G → T
3	ΔF508/ΔF508
4	ΔF508/unidentified
5	ΔF508/ΔF508
6	ΔF508/ΔF508
7	G178R/unidentified

CFTR, cystic fibrosis transmembrane conductance regulator.

emphysema, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, and scleroderma. The CFTR genotype of the CF tissues was determined by allele-specific hybridization (performed at Genzyme, Framingham, MA). All cells were isolated from second through sixth generation bronchi in both wild-type CFTR-expressing (non-CF) and CF HBE cells. The bronchi were incubated overnight at 4°C in MEM containing 0.1% protease XIV, 0.01% deoxyribonuclease, and 1% fetal bovine serum (FBS). The epithelial cells were removed from the underlying musculature by blunt dissection, isolated by centrifugation, and washed in MEM containing 5% FBS. After centrifugation, the cells were resuspended in bronchial epithelial growth medium (Clonetics, San Diego, CA). The cells were then plated into human placental collagen-treated T-25 tissue culture flasks. On reaching 80–90% confluence, the cells were trypsinized (0.1%), resuspended in MEM plus 5% FBS, and seeded onto human placental collagen-coated Costar Transwell filters (0.33 cm^2) at a density of $\sim 2 \times 10^6/cm^2$. After 24 h, the medium was changed to DMEM-F-12 medium (1:1) plus 2% Ultroser G (BioSepra), and an air interface at the apical membrane was established. The medium bathing the basolateral surface was changed every 48 h. Measurements of I_{sc} were performed after ~10–20 additional days in culture.

I_{sc} measurements. Costar Transwell cell culture inserts were mounted in Costar Ussing chambers, and the cultures were continuously short-circuited with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance was measured by periodically applying a 2-mV bipolar pulse and calculated with Ohm's law. The bath solution contained (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, and 10 glucose. The pH of this solution was 7.4 when gassed with a mixture of 95% O_2-5% CO_2 at 37°C. The amiloride-sensitive I_{sc} was taken as a measure of net electrogenic Na^+ transport.

Materials. Aprotinin, SBTI, α_1-AT, trypsin, and amiloride were from Sigma and were dissolved in phosphate-buffered saline (PBS) at 1,000-fold the required experimental concentrations. BAY 39-9437 is a 170-amino acid human serine protease inhibitor (7, 17) that was recombinantly expressed in Chinese hamster ovary (CHO) cells. The secreted protein was chromatographically purified from the culture medium.

Data analysis. All data are presented as means ± SE; *n* is the number of experiments. Apparent inhibitory constant (K_i) values were obtained with nonlinear curve-fitting routines in SigmaPlot (Jandel Scientific, San Rafael, CA). Statistical analysis was performed with Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

The studies reported here were performed on HBE cultures with cells derived from five patients expressing wild-type CFTR (non-CF cells) and seven CF patients (CF cells). Two of the non-CF patients were diagnosed with emphysema or COPD, one with idiopathic pulmonary fibrosis, one with fibrotic connective tissue disease, and one with scleroderma. The CFTR genotype of the seven CF patients is given in Table 1. All seven patients had at least one ΔF508 CFTR allele and three were homozygous ΔF508 CFTR. In total, we evaluated 128 non-CF HBE cultures and 167 CF HBE cultures under I_{sc} conditions. The baseline I_{sc} and transepithelial resistance of non-CF HBE cultures were 35.6 ± 0.90 $\mu A/cm^2$ and 733 ± 14.7 $\Omega \cdot cm^2$, respectively. The I_{sc} and transepithelial resistance of the CF HBE cultures were 42.4 ± 1.13 $\mu A/cm^2$ and 684 ± 15.0 $\Omega \cdot cm^2$, respectively. As in a previous study by Devor et al. (9), amiloride inhibited a greater portion of the I_{sc} in CF cells (82%) than in non-CF cells (70%). These results together with the higher I_{sc} in CF cells versus non-CF cells demonstrate a significant Na^+ hyperabsorption by the CF HBE cultures.

Effects of protease inhibitors on Na^+ transport across HBE cultures. In the first series of experiments, we investigated the effects of several protease inhibitors for their potential effects on Na^+ transport across HBE cultures. A baseline I_{sc} was first measured after a 20-min equilibration period, at which time the protease inhibitor was added to the mucosal bath. After 90 min, the I_{sc} was again recorded, and amiloride (10 μM) was then added to the mucosal bath. After an additional 5 min, the I_{sc} was again recorded. Figure 1 summarizes the results of the effects of several protease inhibitors on the I_{sc} across CF cells with the above protocol. Control cultures were given an equivalent volume of PBS and studied in parallel with the protease-treated cultures. The I_{sc} of the PBS-treated cultures was stable over a 90-min period. Amiloride caused an inhibition of 34 ± 3.8 $\mu A/cm^2$ (n = 29) in the PBS-treated cultures.

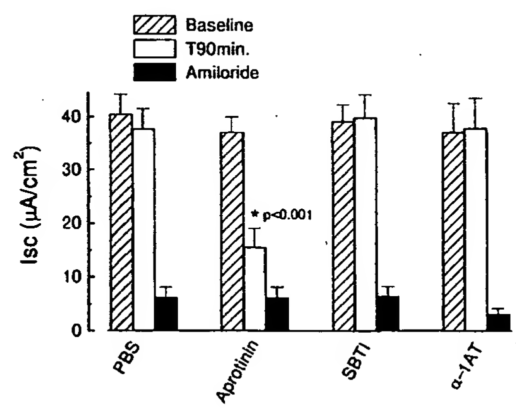

Fig. 1. Effects of various protease inhibitors on cystic fibrosis (CF) human bronchial epithelial (HBE) cell Na^+ transport. The short-circuit current (I_{sc}) of CF epithelial cultures under continuous short-circuit conditions was measured at *time 0* (baseline), 90 min after the addition of PBS or protease inhibitor to the mucosal solution (T90min), and after the addition of amiloride. Drug concentrations were 1 μM aprotinin, 10 μM soybean trypsin inhibitor (STBI), 1 μM α_1-antitrypsin (α_1-AT), and 10 μM amiloride. Values are means ± SE for 29 PBS-treated cultures and 9 cultures for each of the inhibitors. *$P < 0.001$ for aprotinin-treated vs. PBS-treated control cultures.

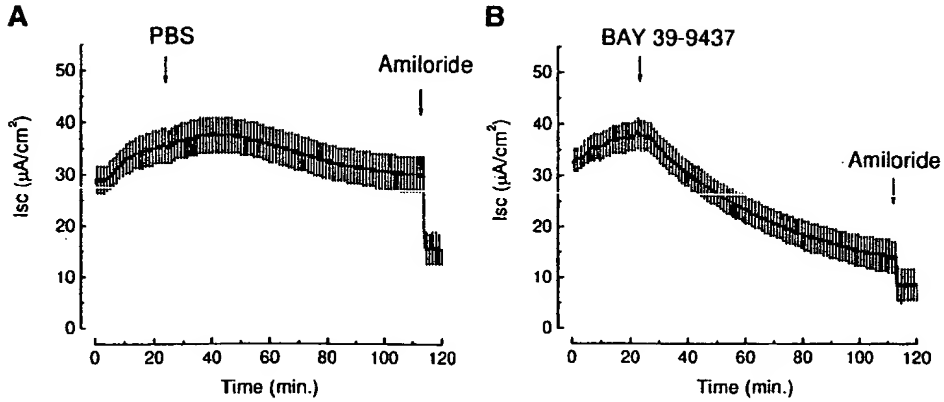

Fig. 2. Effect of BAY 39-9437 on Na^+ transport in non-CF HBE cells. PBS (*A*) or BAY 39-9437 (470 nM; *B*) was added to the mucosal side, and I_{sc} was monitored for 90 min before the addition of amiloride (10 μM) to the mucosal solution. Vertical deflections are the current response to 2-mV bipolar pulses.

In contrast, aprotinin reduced the I_{sc} from a baseline value of 37 ± 2.9 μA/cm² to a value of 15 ± 3.5 μA/cm² (n = 9) after 90 min and amiloride caused a further inhibition of 9.3 ± 3.9 μA/cm². Thus aprotinin caused a significant inhibition in the amiloride-sensitive I_{sc} compared with the PBS-treated control cultures (P < 0.001). SBTI and α_1-AT did not inhibit the I_{sc} or alter the amiloride-sensitive current (Fig. 1). Similar results were obtained with cells from non-CF patients.

These results demonstrate that Na^+ transport in HBE cells is regulated by an aprotinin-sensitive mechanism as previously shown in studies on renal epithelial cells and ENaC expression studies in *Xenopus* oocytes (6, 27, 28). Aprotinin is a serine protease inhibitor of bovine origin and a potent inhibitor of trypsin (11, 12). Although SBTI and α_1-AT are also serine protease inhibitors, only aprotinin contains a Kunitz domain, the protease-inhibitory active site. Reasoning that the Kunitz domain was the essential feature of the inhibitory effect of aprotinin on Na^+ transport, we searched for a human Kunitz-type protease inhibitor. BAY 39-9437 is such a protein and is further described in DISCUSSION. In *BAY 39-9437 inhibition of HBE Na^+ transport*, we document the inhibitory effects of BAY 39-9437 on Na^+ transport across non-CF and CF HBE cell cultures.

BAY 39-9437 inhibition of HBE Na^+ transport. Figures 2 and 3 illustrate the time-dependent inhibition of I_{sc} caused by BAY 39-9437 in non-CF and CF cells, respectively. BAY 39-9437 (470 nM) caused the I_{sc} to decrease, with a half-life ($t_{1/2}$) of ~45 min, whereas the PBS-treated control cells showed little or no change in I_{sc} over a 90-min period. After 90 min, amiloride caused a greater inhibition of the 90-min I_{sc} in the PBS-treated control cells compared with the BAY 39-9437-treated cells. Figure 4 summarizes the results of 25–38 experiments performed as illustrated in Figs. 2 and 3. Over a 90-min period, BAY 39-9437 (470 nM) inhibited a significant portion of the amiloride-sensitive I_{sc} in both non-CF (68 ± 3.3%) and CF cells (72 ± 3.9%) compared with the time-dependent changes in the PBS-treated control cells run in parallel (P < 0.001). The inhibition in I_{sc} by BAY 39-9437 was observed with cells from all five non-CF patients and all seven CF patients.

The effects of BAY 39-9437 on I_{sc} were concentration dependent in all patients as illustrated in Fig. 5 for studies on CF cells from *patient 4*. The values shown in Fig. 5 are the percent change in I_{sc} at 90 min after correction for the amiloride-insensitive I_{sc}. BAY 39-9437 at 4.7 nM did not significantly alter the I_{sc}. However, at 16 nM, BAY 39-9437 caused a significant inhibition in the I_{sc}, suggesting that the minimal effective concentration falls between 4.7 and 16 nM. The half-maximal effective concentration ($K_{1/2}$) for the studies shown in Fig. 5 was 25 ± 7.9 nM and a maximal inhibition of 87 ± 6.4% when fit to a simple Michaelis-Menten inhibition function. The inhibition of I_{sc} by

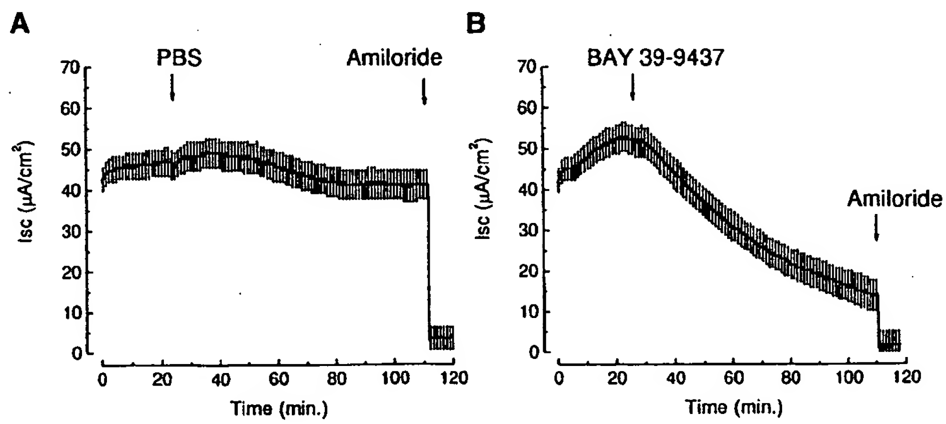

Fig. 3. Effect of BAY 39-9437 on Na^+ transport in CF HBE cells. PBS (*A*) or BAY 39-9437 (470 nM; *B*) was added to the mucosal side, and I_{sc} was monitored for 90 min before the addition of amiloride (10 μM) to the mucosal solution. Vertical deflections are the current response to 2-mV bipolar pulses.

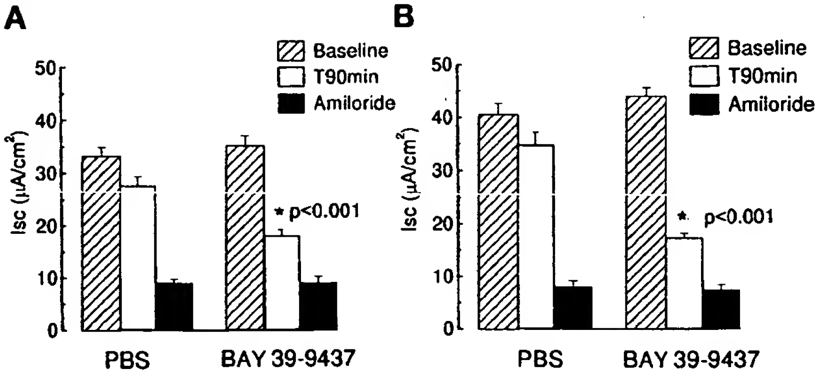

Fig. 4. Effects of PBS and BAY 39-9437 on non-CF (*A*) and CF (*B*) HBE cell Na^+ transport. Studies were performed as in Fig. 2. Drug concentrations were 470 nM BAY 39-9437 and 10 μM amiloride. Values are means ± SE; n = 25–35 filters. *P < 0.001 for BAY 39-9437-treated vs. PBS-treated control cells.

BAY 39-9437 in non-CF cells from a patient diagnosed with emphysema or COPD was also concentration dependent, with a very similar $K_{1/2}$ of 24 ± 8.5 nM but with a lower maximal inhibition of 54 ± 2.8%. The lower maximal inhibition in non-CF cells compared with CF cells may reflect the contribution of anion secretion to the I_{sc} in non-CF cells.

The inhibitory effects of BAY 39-9437 were completely reversed by the addition of trypsin to the mucosal solution (Fig. 6*B*). In the experiment shown in Fig. 6, BAY 39-9437 (470 nM) decreased the I_{sc} from a baseline value of 66 μA/cm² to a value of 26 μA/cm² over a 90-min period. Amiloride (10 μM) further inhibited the I_{sc} to a value of 6 μA/cm². An exchange of the mucosal bath with BAY 39-9437 and amiloride-free buffer restored the I_{sc} to the preamiloride I_{sc} value. The addition of trypsin (1 μM) to the mucosal bath caused a rapid increase in the I_{sc} to the pre-BAY 39-9437 (baseline) level of 70 μA/cm², and this current was nearly completely inhibited by amiloride. These results are representative of nine similar experiments with CF cells in which trypsin increased the I_{sc} to 93 ± 7% of the pre-BAY 39-9437 level. The addition of trypsin to the mucosal bath of PBS-treated cells had little or no effect on the I_{sc} (Fig. 6*A*). Similar results were obtained with PBS- and BAY 39-9437-treated non-CF cells (data not shown).

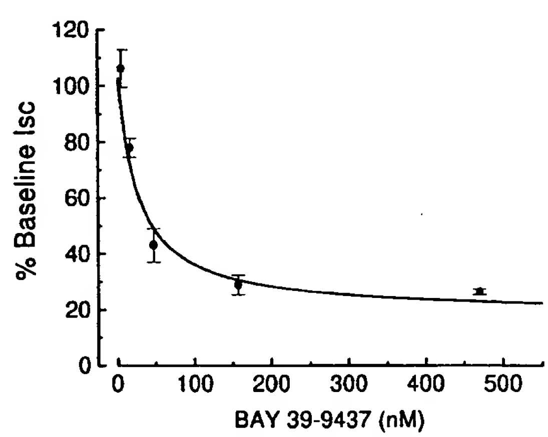

Fig. 5. Concentration-dependent effects of BAY 39-9437 on Na^+ transport in CF HBE cells. Values are percent change in I_{sc} at 90 min corrected for the amiloride-insensitive current; n = 5 filters/group. BAY 39-9437 was added to the mucosal solution at *time 0* at the indicated concentrations, and amiloride (10 μM) was added at 90 min. Solid line, fit to a simple Michaelis-Menten inhibition function. The PBS-treated control cells for this series of experiments had a mean baseline I_{sc} of 30.4 ± 2.8 μA/cm² that was at 107 ± 7% at 90 min.

In a second series of experiments designed to investigate the reversible inhibition of Na^+ transport by BAY 39-9437, CF cells were incubated overnight with 25 μl of PBS or PBS plus 470 nM BAY 39-9437 on the apical surface. The cells were then washed and placed in Ussing chambers, and the I_{sc} was monitored. Overnight treatment with BAY 39-9437 caused a similar degree of inhibition in Na^+ transport as observed after a 90-min treatment compared with that in PBS-treated cells (PBS treated: 50.8 ± 2.6 μA/cm², n = 25; BAY 39-9437 treated: 15.5 ± 2.8 μA/cm², n = 14). However, in contrast to the PBS-treated cells, the BAY 39-9437-treated cells showed a steady rise in I_{sc} over a 45-min period to a new higher plateau value that was nearly equal to the initial I_{sc} of the PBS-treated cells (Fig. 7). The addition of trypsin (1 μM) to the mucosal bath caused little change in the I_{sc} of PBS-treated cells (<2.5 ± 1.3 μA/cm²; n = 9) as previously shown in Fig. 6. In contrast, trypsin caused a rapid rise in the I_{sc} of BAY 39-9437-treated cells, and the magnitude of this increase was greater in cells left for 15 min compared with cells left for 45 min (Fig. 7, *B* and *C*, respectively). The results shown in Fig. 7 are representative of six similar experiments. Trypsin added at 15 min increased the I_{sc} by 27 ± 60 μA/cm² and at 45 min by only 12 ± 4.2 μA/cm² in the BAY 39-9437-treated cells. These results demonstrate that the effects of BAY 39-9437 are reversible after a wash, with a $t_{1/2}$ of ~15 min, and suggest that the activity of an endogenous protease is preserved after BAY 39-9437 treatment and washout.

DISCUSSION

BAY 39-9437 is a recombinant human serine protease inhibitor. The natural protein is a 252-amino acid protein composed of a signal peptide, two protease-binding Kunitz domains, a transmembrane domain, and an intracellular domain. This protein was originally identified and isolated from the placenta and is referred to as placental bikunin (7, 17). Northern blot

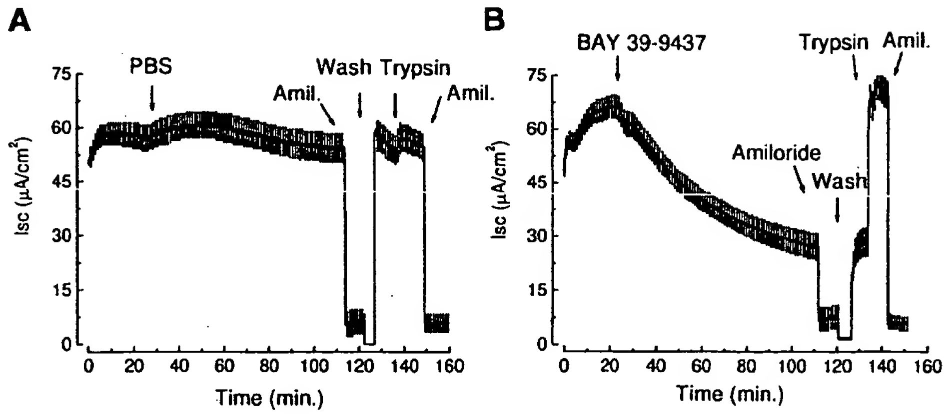

Fig. 6. Time course and reversal by trypsin of the inhibitory effect of BAY 39-9437 (*B*) on Na^+ transport in CF HBE cells. *A*: PBS treatment. Drug concentrations were 470 nM BAY 39-9437, 10 µM amiloride (Amil), and 1 µM trypsin, and drugs were added to the mucosal solution at the indicated times (arrows). After the 1st addition of amiloride, the mucosal bath was exchanged with a 20× volume (100 ml) of amiloride- and BAY 39-9437-free buffer. Vertical deflections are the current responses to 2-mV pulses.

analysis demonstrated the expression of placental bikunin in pancreas, kidney, brain, heart, and lung. The gene for placental bikunin was mapped to chromosome 19q13 (17), and it is noteworthy that a CF modifier gene has been mapped to this same locus (33). Loss of function mutations in placental bikunin are expected to increase the severity of CFTR disease, causing mutations, whereas an increase in the expression or activity of placental bikunin should diminish the severity of CFTR disease, causing mutations. The recombinant product BAY 39-9437 is a protein of 170 amino acid residues. BAY 39-9437 was expressed in CHO cells as a secreted form of placental bikunin lacking the transmembrane and intracellular domains such that it is composed of the signal peptide and the two extracellular Kunitz domains. Both placental bikunin and BAY 39-9437 inhibit trypsin (K_i = 0.01 nM), plasmin (K_i = 0.1 nM), and kallikrein (K_i = 0.3 µM) at a 2:1 enzyme-to-inhibitor binding stoichiometry (7, 17). However, placental bikunin and BAY 39-9437 do not inhibit urokinase, tissue plasminogen activator, or elastase at concentrations up to 1 µM (7, 17).

The studies reported here demonstrate that BAY 39-9437 is a potent inhibitor of electrogenic Na^+ transport in HBE cells. The Na^+ transport inhibitory effects of BAY 39-9437 develop slowly, with a $t_{½}$ of ~45 min (Figs. 2, 3, and 6), and are reversible on washing, with a $t_{½}$ of ~15 min (Fig. 7), or develop rapidly with the addition of trypsin (Figs. 6 and 7). The inhibitory effects of BAY 39-9437 on Na^+ transport were concentration dependent and well described by a simple Michaelis-Menten inhibition function, with a $K_{½}$ of ~25 nM in both non-CF and CF cells. These results do not preclude the possibility that both Kunitz domains participate in the inhibition of Na^+ transport. However, they do suggest that there is no cooperativity between the two domains if indeed both Kunitz domains participate. BAY 39-9437 (470 nM) caused approximately the same degree of inhibition in the amiloride-sensitive I_{sc} in non-CF (68%) and CF (72%) cells. The inhibitory effect of BAY 39-9437 was nearly completely reversed after a wash with an inhibitor-free solution. I_{sc} increased, with a $t_{½}$ of ~15 min, after removal of BAY 39-9437, suggesting that the activity of the ENaC-activating protease was preserved after BAY 39-9437 treatment. The inhibition of Na^+ transport by BAY 39-9437 could also be rapidly reversed by the addition of trypsin to the mucosal bath. Mucosal trypsin had no effect on the baseline I_{sc} or the 90-min I_{sc} in PBS-treated non-CF or CF HBE cells. Only after inhibition with BAY 39-9437 was the stimulation of an amiloride-sensitive I_{sc} with trypsin observed.

Our working hypothesis for the protease-mediated modulation and inhibition of HBE Na^+ transport by BAY 39-9437 is illustrated in Fig. 8. As originally suggested for the modulation of ENaC in renal epithelia (6, 27, 28), we propose that there is a CAP1-like protease in the apical membrane of HBE cells. ENaC is inserted into the apical membrane as an inactive or partially active channel where it is activated by an apical membrane CAP1-like protease. Active ENaC remains in the apical membrane for a $t_{½}$ of ~45 min when it is then retrieved, probably ubiquitinated, and degraded (25). The addition of a CAP1 inhibitor such as BAY 39-9437 prevents the activation of ENaC by CAP1. The addition of an exogenous protease such as trypsin can circumvent the inhibition of CAP1 and cause the activation of ENaC.

The molecular identity of the HBE cell CAP1-like protease remains to be established, but one possible candidate is prostasin (30–32). In support of this notion, prostasin shares an 80% homology with mCAP1 and 50% homology with xCAP1. Moreover, prostasin was shown to be inhibited by aprotinin but not by SBTI (30), results consistent with the inhibition of Na^+ transport by aprotinin but not by SBTI (Fig. 1). The prostatin gene (PRSS8) has been localized to chromosome 16p11.2 (21) and is another potential gene that could act as a CF modifier gene. Prostasin is synthesized by the cell as a preproenzyme and may remain on the plasma membrane or be released from the cell surface as a secreted protein (30, 31). The results shown in Fig. 7 suggest that the protease responsible for the activation of ENaC in HBE cells is not readily washed off and thus may remain as a membrane-anchored protein. The enzymes involved in the synthesis of prostasin and other CAP1-like proteases are unknown. The substrate for the CAP1-like proteases is also unknown. Initial experiments suggest that ENaC may not be the CAP1 substrate (6, 27). However, it

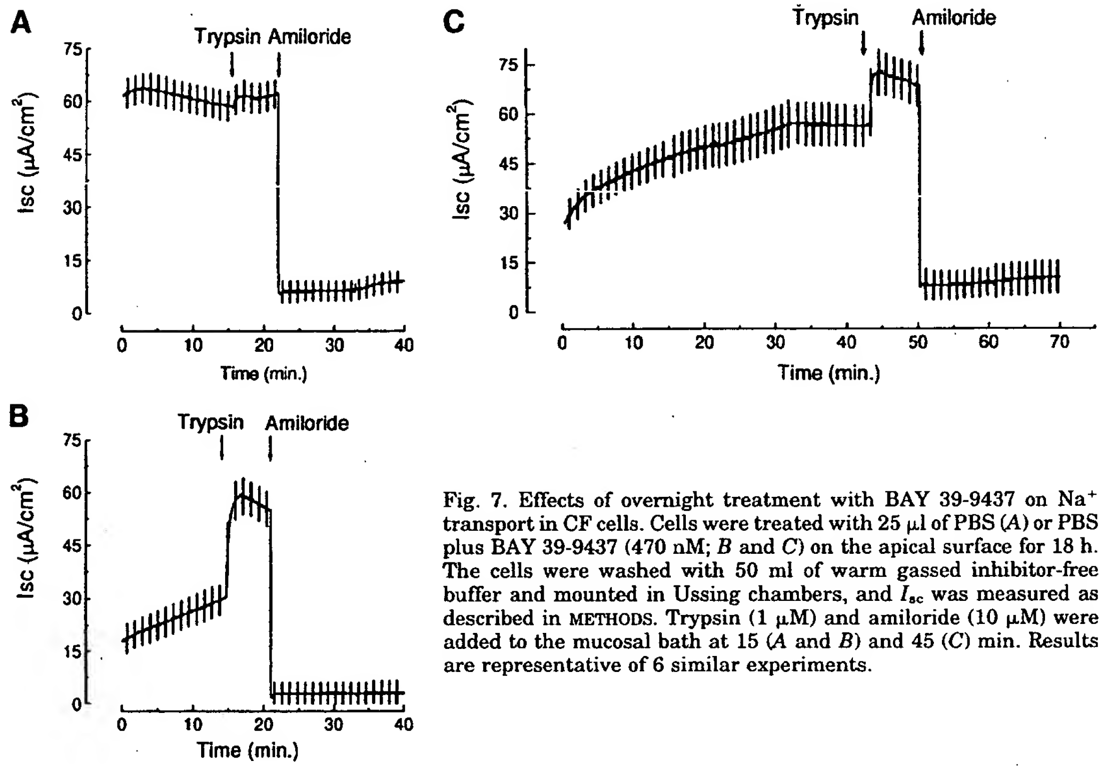

Fig. 7. Effects of overnight treatment with BAY 39-9437 on Na^+ transport in CF cells. Cells were treated with 25 μl of PBS (*A*) or PBS plus BAY 39-9437 (470 nM; *B* and *C*) on the apical surface for 18 h. The cells were washed with 50 ml of warm gassed inhibitor-free buffer and mounted in Ussing chambers, and I_{sc} was measured as described in METHODS. Trypsin (1 μM) and amiloride (10 μM) were added to the mucosal bath at 15 (*A* and *B*) and 45 (*C*) min. Results are representative of 6 similar experiments.

remains possible that ENaC is a protease-activated channel. If ENaC is not the CAP1 substrate, it will be important to identify the substrate as well as the signal transduction cascade that leads to the activation of ENaC. Given the emergence of protease-activated receptors (PARs) that are G protein coupled to intracellular signal transduction cascades (8), one may speculate that CAP1 acts on a novel PAR to cause the activation of ENaC. Clearly, additional studies are necessary to determine whether ENaC is a protease-activated channel or to identify the CAP1-activated PAR involved in the regulation of ENaC. *Xenopus* oocyte expression studies (6, 27) suggest that CAP1 activation of ENaC involves an increase in the channel open probability and not an increase in the number of channels, results that are consistent with the model shown in Fig. 8. Prolonged overnight incubation with aprotinin or BAY 39-9437 did not completely inhibit the amiloride-sensitive I_{sc} in HBE cells (Fig. 7). These results suggest that ENaC may be inserted into the

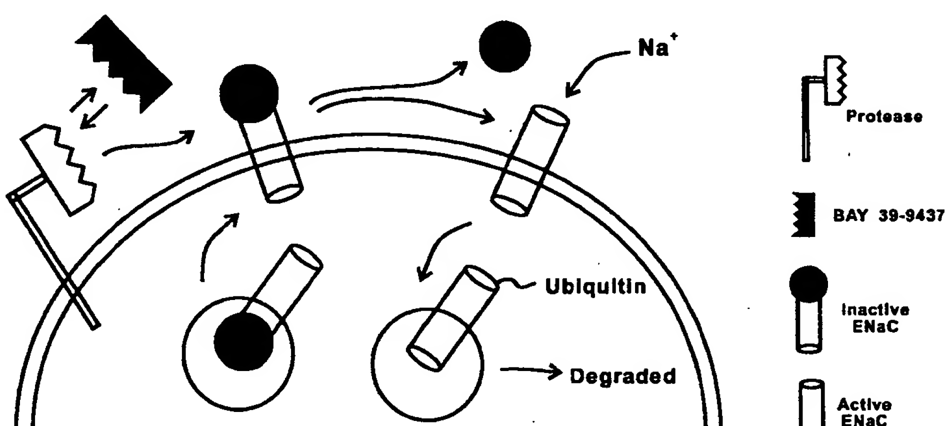

Fig. 8. Working hypothesis for the protease-mediated regulation of Na^+ transport in HBE cells. Inactive epithelial Na^+ channel (ENaC) is inserted into the apical membrane where it is activated by an apical membrane, extracellular BAY 39-9437-inhibitable protease. Active ENaC is removed from the apical membrane with a half-life of 45 min. Retrieved ENaC is probably ubiquitinated and then degraded. Proteolytic activation of ENaC can be blocked by extracellular BAY 39-9437 without altering the insertion of new inactive ENaC, which can subsequently be activated by the addition of exogenous trypsin. The substrate of the channel-activating protease or the exogenous trypsin is unknown and may be ENaC or some regulatory protein closely associated with ENaC.

apical membrane as a partially active channel, that is, a channel with a finite but low open probability. CAP1-mediated activation then leads to an increase in the channel open probability. Fluctuation analysis to obtain estimates of channel density and open probability could be used to investigate the mechanisms involved in the protease-mediated modulation of ENaC activity.[1]

Although controversy abounds in the CF research community regarding the composition and volume of the airway surface liquid in normal and CF airways (29), there is unanimous agreement that improving the clearance of mucus in CF patients would be of major therapeutic benefit. Indeed, impaired mucociliary clearance is a clinical feature of many airway diseases including CF (20, 23). The result of impaired mucociliary clearance is mucus retention and accumulation that appear to contribute to the severity of the disease. The inhibition of Na^+ transport is anticipated to improve mucociliary clearance. Support for this logic is seen in another genetic disease, pseudohypoaldosteronism, caused by loss of function mutations in ENaC (5, 15, 26). These patients display a fourfold increase in mucociliary transport compared with control patients (14). Several attempts have been made with amiloride to show the therapeutic benefit of inhibiting Na^+ transport in CF patients (4, 13, 16). However, the results of these trials have been disappointing. The weak affinity of amiloride for ENaC (K_i = 300 nM) and the rapid clearance of this low molecular mass compound from the airways (1, 18, 19) are thought to be important reasons for the poor efficacy of amiloride. The in vitro studies reported here indicate that BAY 39-9437 has a 10-fold improved potency for the inhibition of ENaC compared with amiloride (25 vs. 300 nM). Moreover, as a 21-kDa recombinant protein, BAY 39-9437 is expected to have a longer residence time in the airways compared with that of amiloride. Thus if BAY 39-9437 can be delivered to the airways, it should inhibit Na^+ transport and thereby improve mucociliary clearance. Animal studies reported by Newton et al. (21) document a decrease in the tracheal transepithelial potential difference caused by the local instillation of BAY 39-9437, results consistent with the inhibition of Na^+ transport. Most importantly, BAY 39-9437 caused a twofold increase in tracheal mucociliary clearance. Thus we are optimistic that the human recombinant serine protease inhibitor BAY 39-4937 may be of therapeutic benefit in the treatment of CF.

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[1]An alternative hypothesis that has not been thoroughly addressed by the present or previous studies is the possible direct inhibition of ENaC by the Kunitz-type protease inhibitors. A number of studies (e.g., Refs. 10, 24) have demonstrated the inhibition of K^+ channels by Kunitz-type protease inhibitors, and this possibility remains to be formally excluded for the action of aprotinin and BAY 39-9437 on ENaC.

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